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REGULATION AND PROPERTIES OF RICE ASPARTOKINASE[1,2]

JEI-FU SHAW and CHUN-YING KU

Institute of Botany, Academia Sinica
Nankang, Taipei, Taiwan 115, Republic of China

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Abstract

Aspartokinase from rice-seedling extract was only inhibited 47% by 10 mM L-lysine and not affected by other amino acids tested, whereas the aspartokinase from milky-stage rice leaves was inhibited to various extent by lysine, threonine, methionine and isoleucine either alone or in combination. Aspartokinase from milky-stage leaf sheaths was totally insensitive to all amino acids tested.

The lysine sensitive aspartokinase from rice seedlings was purified 16-fold by ammonium sulfate fractionation, Sephacryl S-300 and DEAE-Sephadex A-50 chromatography. The optimum pH was 7.5 and the optimum temperature was 35°C. The Km values for L-aspartate and $MgATP^{2-}$ were 1.37 mM and 0.55 mM respectively. The lysine concentration for half maximal inhibition was 44 μM and the Hill coefficient was 2.8. The thermal inactivation of the aspartokinase followed first order reaction kinetics. Lysine stabilized the enzyme against inactivation. At pH 7.5 and 45°C, the inactivation rate constants of the enzyme in the absence of lysine was 0.042 min^{-1}, whereas in the presence of 1 mM lysine, there were two inactivation rate constants, 0.024 min^{-1} and 0.005 min^{-1}.

Introduction

Aspartokinase (ATP: L-aspartate 4-phosphotransferase, E.C. 2.7.2.4.) catalyzes the first reaction in the biosynthetic pathway of aspartate family amino acids, lysine, methionine, threonine and isoleucine in bacteria and higher plants. Since the pathway is branched, the end product regulation mechanism, molecular properties and kinetic mechanisms have been the subject of great research interest (Miflin, 1977; Shaw *et al.*, 1983; Shaw, 1979; Shaw and Smith, 1976, 1977; Truffa-Bachi, 1973). Several regulation mechanisms including concerted feedback inhibition and isozymes sensitive to different end products have been proposed in different organisms.

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It has been found that poor growth of rice callus tissue was observed on methionine deficient media containing threonine and lysine, homoserine supplementation relieved the growth inhibition (Furuhashi and Yatazawa, 1970). Studies on the rice aspartokinase properties would provide insight on the regulation mechanism of rice growth by these amino acids. So far there were no reports on rice aspartokinase in these respects.

In the present work, we have partially purified the rice-seedling aspartokinase, studied its properties, and compared the regulation patterns of aspartokinase from rice seedlings and milky-stage leaves. These studies would provide some clues for understanding the role of aspartokinase in rice amino acid biosynthesis and regulation.

Materials and Methods

Chemicals

Adenosine 5′-triphpsphate (disodium salt, from equine muscle) and Good's buffer (PIPES, HEPES, CAPS, Bis-Tris) were obtained from Sigma Chemical Co. Ammonium sulfate and amino acids were obtained from E. Merck Co. All other reagents were of ACS reagent grade.

Plant Materials

Rice seeds (*Oryza sativa* L. var. Shingchu 56) were surface sterilized by 1% sodium hypochlorite for 30 minutes and were germinated in the dark in moist vermiculite at 27°C for two weeks.

Extraction of Enzyme

Freshly excised shoots were homogenized at 4°C in polytron homogenizer with buffer (50 mM potassium phosphate buffer, pH 7.5, containing 0.5 mM lysine, 1 mM EDTA, 10 mM β-mercaptoethanol, 20% (v/v) glycerol in a volume of 2 ml/g, 2% polyvinyl polypyrolidone and 5 drops of octanol were added to the extraction buffer to remove phenolics and prevent foaming). The homogenates were squeezed through four layers of cheesecloth and centrifuged at 16,000 g for 30 minutes. The resulting supernatants were subjected to ammonium sulfate fractionation. Proteins precipitating between 40 and 63% saturation $(NH_4)_2SO_4$ fraction were collected by centrifugation, dissolved in a minimal volume of the same buffer and then applied to a Sephacryl S-300 column (2.5×47 cm), and eluted with the same phosphate buffer (pH 7.5). The fractions containing aspartokinase activity were pooled and subjected to DEAE-Sephadex A-50 column (2.5×35 cm) chromatography and eluted with linear gradient of the same phosphate buffer containing NaCl salt (0.1–1.0 M). The fractions of aspartokinase were then collected and concentrated by Millipore immersible CX-30™ ultrafiltration unit.

Enzyme Assays

Aspartokinase activity was measured by hydroxamate assay. The reaction mixture contained 20 mM ATP, 50 mM L-aspartate, 10 mM β-mercaptoethanol, 100 mM Tris-HCl buffer (pH 7.5), 20 mM $MgSO_4 \cdot 7H_2O$ and 400 mM KOH-neutralized $NH_2OH \cdot HCl$. All reagents were adjusted to pH 7.5 with KOH. The total volume was 1 ml. The reaction was initiated by the addition of enzyme and after 2 h the reaction was terminated by adding 0.5 ml of 15% trichloroacetic acid. The denatured protein was removed by a Eppendorf centrifuge. After adding 1 ml of 10% $FeCl_3$-0.7 N HCl to the supernantant, the absorbance at 540 nm was measured by a Hitachi 100-60 spectrophotometer. The enzyme was assayed in triplicate.

Measurement of Protein

The protein content was determined by the method of Lowry *et al.* (1951), using bovine serum albumin as a standard.

Results

The Effect of L-Amino Acids on the Activity of Rice Aspartokinase

We have tested several L-amino acids on the enzyme activities of three different preparations of aspartokinase (Table 1). In the two-week-old rice seedlings, only lysine sensitive aspartokinase was present in either crude extract (a) or purified enzyme (b). There is little effect (if any) of other amino acids either alone or in combination on the lysine sensitive aspartokinase. However, the purified aspartokinase appeared to show increased sensitivity toward lysine inhibition (increased from 47% to 83% inhibition). This suggests that there may be insensitive aspartokinase present in seedling crude extract. For the enzyme of the milky-stage rice plants (100 days after transplantation), there was no significant effect by all the amino acids tested on the aspartokinase in the crude extract from leaf sheath, however, the leaf aspartokinase exhibited various degree of inhibition by the five L-amino acids tested (c in Table 1).

The leaf aspartokinase was partially inhibited by end products lysine, threonine and methionine alone, suggesting isozymes individually sensitive to each amino acid could be present. The different amino acid inhibition patterns between the rice-seedling and milky-stage leaf aspartokinase clearly indicate the changes in the regulation of amino acid metabolism during plant growth and differentiation.

Leucine is an amino acid unrelated to the "aspartic family" amino acid biosynthetic pathway. However, it has been found to activate or inihbit aspartokinase in several cases (Aarnes, 1974). In rice leaf aspartokinase, leucine was a partial inhibitor (Table 1). Therefore, metabolic interactions between different biosynthetic pathways of amino acics were probably present in the rice plant.

Table 1. *The effect of amino acids on the activity of aspartokinase*

Amino acid added	Aspartokinase activity (% of control)		
	a	b	c
None	100	100	100
10 mM Thr	100	100	38
10 mM Lys	53	17	30
10 mM Met	95	103	36
10 mM Leu	—	109	33
10 mM Ile	—	102	38
10 mM Thr+10 mM Lys	55	17	66
10 mM Lys+10 mM Met	52	20	29
10 mM Thr+10 mM Met	89	107	30
10 mM Ile+10 mM Lys	—	25	23
10 mM Ile+10 mM Met	—	105	34
10 mM Thr+10 mM Lys+10 mM Met	64	26	11
10 mM Ile+10 mM Met+10mM Lys	—	24	55

a: Crude enzyme of rice seedling precipitated by 62.5% ammonium sulfate and dialyzed against 50mM potassium phosphate buffer (pH 7.5) including 10mM β-mercaptoethanol, 1 mM EDTA and 20% glycerol.
b: Purified aspartokinase of rice seedlings as described in experimental proceedure.
c: Crude enzyme of milky-stage rice leaves (100 days after transplantation) prepared as (a).

In the leaf enzyme the only combination of amino acids that produced higher inhibition was Thr + Lys + Met. Surprisingly, the aspartokinase activity in the presence of lysine and threonine was significantly less than either amino acid alone. The inhibition of enzyme activity in the presence of isoleucine, methionine and lysine together was also significantly less than either amino acid alone or any two amino acids in combination (c in Table 1). Molecular interactions between amino acids on aspartokinase activity presumably exist to explain these phenomena. Further studies on purified isozymes are needed to elucidate the mechanism.

Partial Purification of Aspartokinase from Seedlings

The rice seedlings appeared to contain very low amount of aspartokinase activity, 1 kg of rice seedlings (fresh weight) only contained 255 unit of aspartokinase (1 unit = $\Delta OD_{540}/2$ h reaction, the molar extinction coefficient of aspartyl hydroxamate is 600, therefore 1 unit is equal to 0.035 μmole of aspartyl phosphate formed/min under the assay condition).

After ammonium sulfate fractionation, Sephacryl S-300 (Fig. 1) and DEAE Sephadex A-50 (Fig. 2), we obtained a 16 fold purification of rice aspartokinase

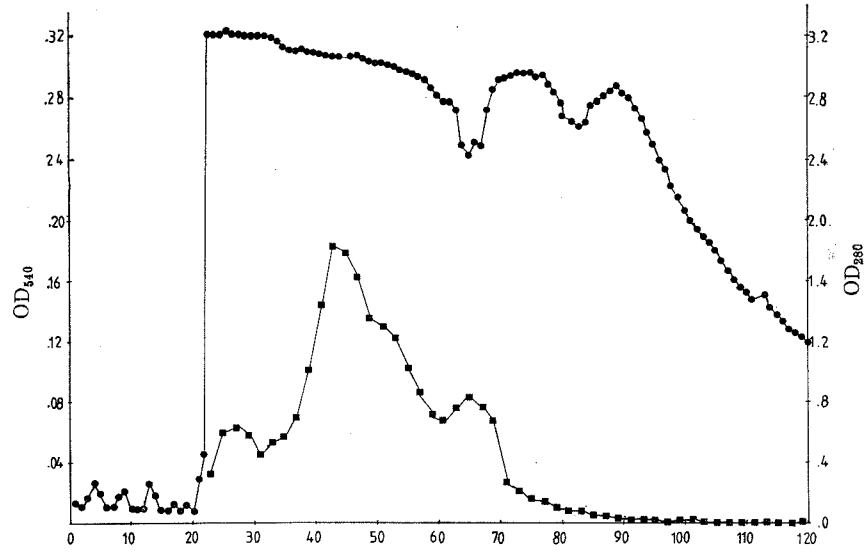

Fig. 1. Sephacryl S-300 chromatograph. Enzymes from ammonium sulfate fractionation (40-62.5% saturation) were dissolved in a minimal volume (14 ml) of potassium phosphate buffer (50 mM pH 7.5) including 0.5 mM lysine, 1 mM EDTA, 10 mM 2-mercapto-ethanol and 20% glycerol, then applied to the column (2.5×57 cm) and eluted with the same buffer at a rate of 9 ml/h. One fraction was 3 ml. Fractions were monitored for aspartokinase activity (■-■, OD_{540}/2 hour reaction) and absorbance at 280 nm (●-●). Fractions 25 to 69 were pooled and concentrated to 70 ml.

and 13% recovery of activity (Table 2). This preparation was used in the subsequent studies unless otherwise indicated. The enzyme was dialyzed to remove salts and lysine before use.

Optimal pH

The aspartokinase was assayed in different Good's buffer to determine its pH-activity profile at 25°C. As shown in Fig. 3, the optimal pH was 7.5 in HEPES

Table 2. *Purification of rice aspartokinase*

Step of purification	Volume (ml)	Aspartokinase activity (units/ml)	Total activity (units)	Protein (mg/ml)	Specific activity (units/mg)	Recovery (%)
1. Crude extract	1000	0.26	255	104.0	0.0024	100
2. 40-62.5% $(NH_4)_2SO_4$ ppt.	35	1.60	56.1	170.2	0.0094	22
3. Sephacryl S-300	170	0.37	63.5	31.1	0.0120	25
4. DEAE-Sephadex A-50	94	0.35	32.9	8.9	0.0394	13

1 unit = $\triangle OD_{540}$/2 h

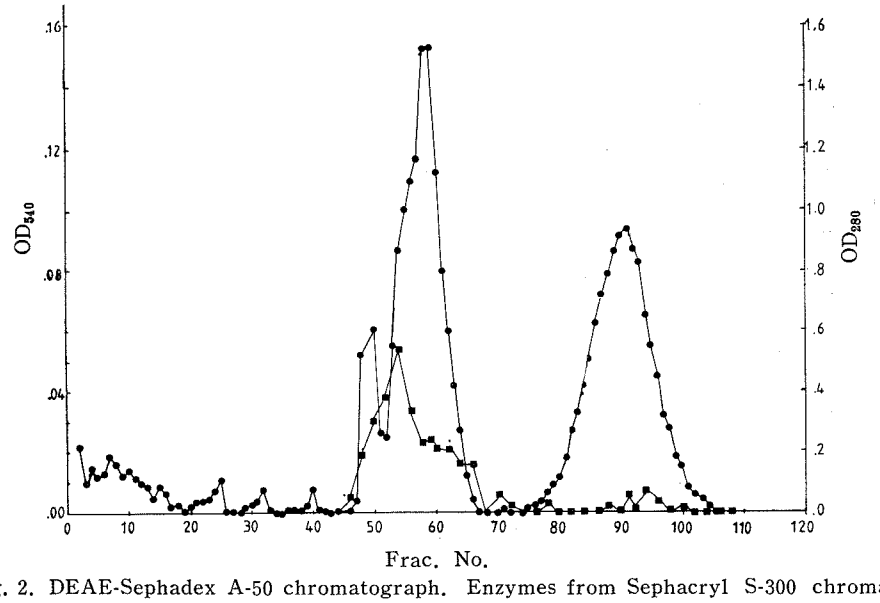

Fig. 2. DEAE-Sephadex A-50 chromatograph. Enzymes from Sephacryl S-300 chromatograph were applied to the column (2.5 × 40 cm) and eluted with a gradient of NaCl (0.1-1 M) in the same potassium phosphate buffer at a rate of 9 ml/h. Fractions (3 ml) were collected and monitored for enzyme activity (■-■) and absorbance at 280 nm (●-●) as described in Fig. 1. Fractions 48 to 66 were pooled and concentrated to 4 ml and stored in freezer.

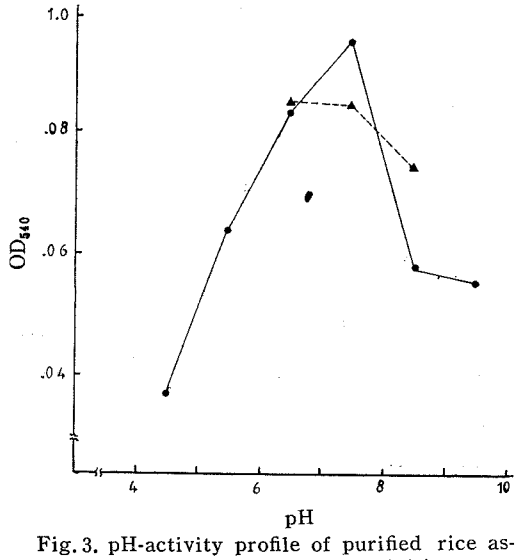

Fig. 3. pH-activity profile of purified rice aspartokinase. Enzyme activities were determined by hydroxamate assay in various pH with all reagents adjusted to the indicated pH by either HCl or LiOH. Buffers used were: pH 4.5-5.5, CH_3COONa; pH 6.5, PIPES (●), pH 7.5, HEPES (●); pH 8.5-9.5, CAPS (●). Bis-Tris buffer was also used in pH 6.5 to 8.5 (▲···▲).

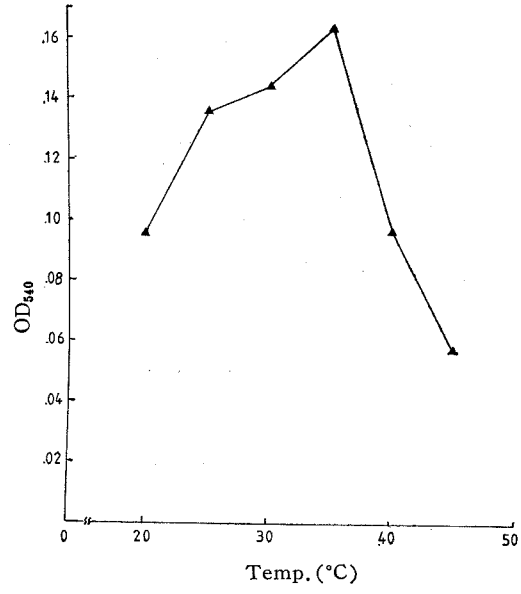

Fig. 4. Temperature-activity profile of purified rice aspartokinases. Enzyme activities were determined by hydroxamate assay at pH 7.5 as described in "Methods" at various temperatures in a water bath.

buffer. However the activity profile was somewhat different using Bis-Tris buffer system.

Optimal Temperature

The enzyme was assayed in 100 mM Tris buffer at pH 7.5 for its temperature-activity profile. The activity rised to the maximum at 35°C and dropped sharply as temperature further increased (Fig. 4).

Kinetic Parameters

When the enzyme was assayed at pH 7.5 and at $[Mg^{++}]/[ATP]=1$, the Km values for L-aspartate and $MgATP^{2-}$ were 1.37 mM and 0.5 mM, respectively. (Figs. 5 and 6).

Inhibition by L-lysine

As shown in Figs. 5 and 6, the lysine was noncompetitive with respect to either aspartate or $MgATP^{2-}$. Fig. 5 shows the sigmoid inhibitory effect of L-lysine on aspartokinase activity. The enzyme used in this experiment was an earlier preparation omitting the Sephacryl S-300 chromatography step, the maximum inhibition was about 60%. From the Hill plot (log $[(v_o-v_i)/(v_i-v_s)$ vs. log $[lys]$. where v_o, v_i, v_s

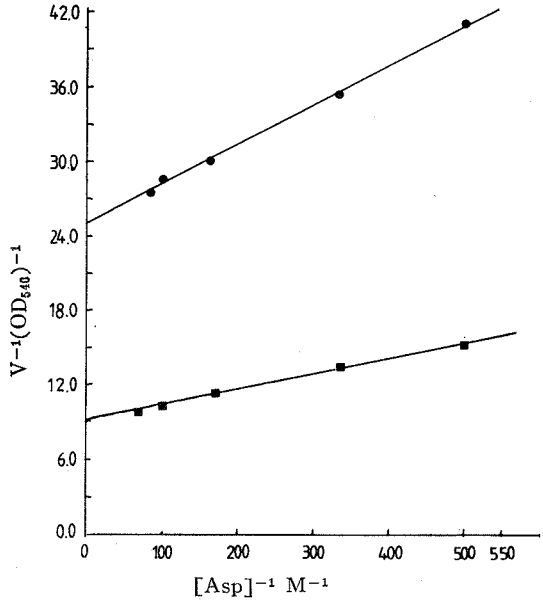

Fig. 5. The inhibitory effect of L-lysine with respect to L-aspartate. Hydroxamate assay with 20 mM MgA TP and various concentrations of L-aspartate was used to measure purified rice aspartokinaes activity. Enzyme activity is expr essed as the absorbance at 540 nm in two hour reaction. (■), no lysine; (●), 0.08 mM lysine.

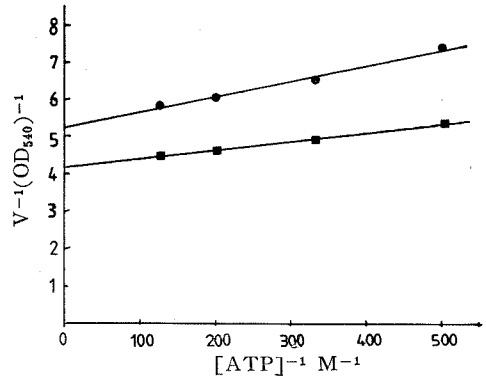

Fig. 6. The inhibitory effect of L-lysine with respect to MgATP. Enzyme assay conditions are the same as in Fig. 5, except the varied substrate is MgATP and the aspartate concentration was kept constant at 50 mM. (■), 0.08 mM L-lysine;(●), no lysine.

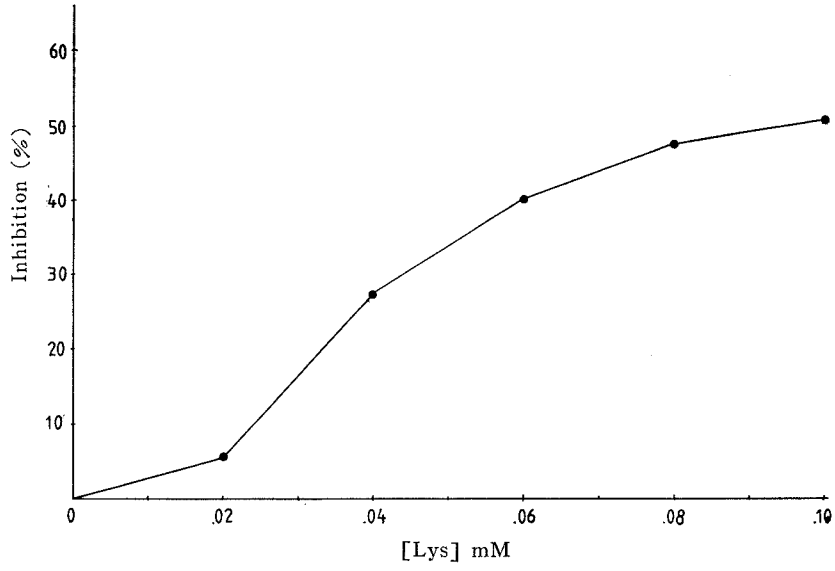

Fig. 7. The lysine inhibition curve. Rice aspartokinase purified without Sephacryl S-300 chromatography step was used in this experiment. Enzyme was assayed in the presence of various lysine concentrations using standard hydroxamate assay.

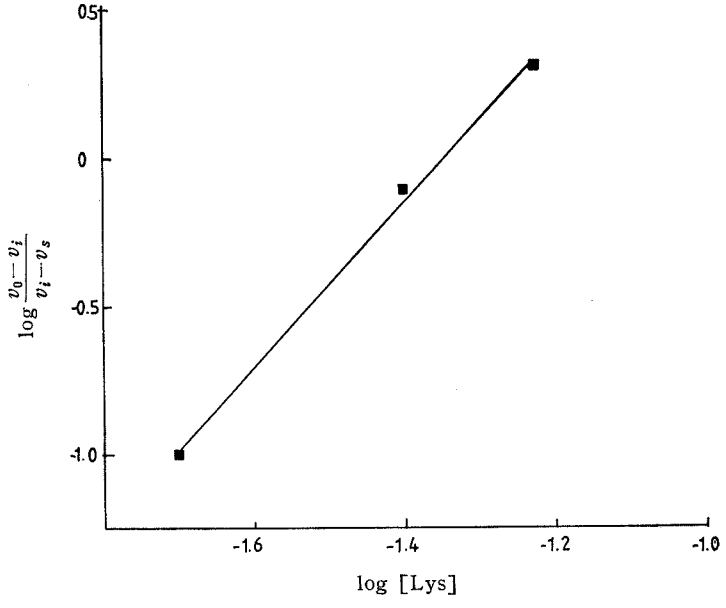

Fig. 8. Hill plot for lysine inhibition. Data from Fig. 7 were used for log $(v_0-v_i)/(v_i-v_s)$ vs. log [Lys] plot, where v_0, v_i, v_s represent enzyme activities in the absence of, in the presence of non-saturating and saturating concentration of lysine, respectively.

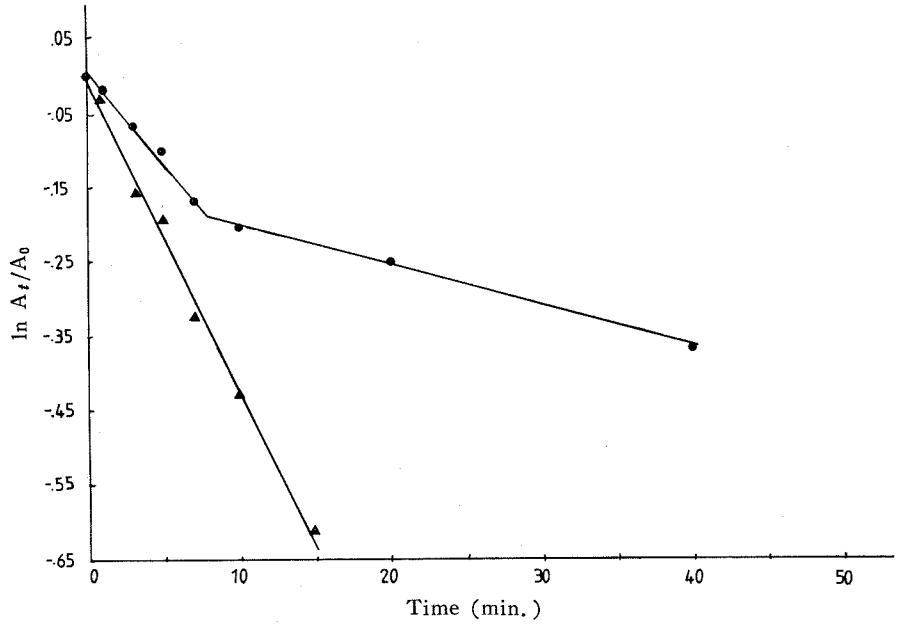

Fig. 9. Thermal inactivation of rice aspartokinase. Enzymes in potassium phosphate buffer (pH 7.5) with (●) and without (▲) 1 mM L-lysine were incubated in a water bath at 45°C for various time intervals. The residual activities (A_t) were assayed as described in "materials and methods" and compared with unheated control (A_0).

represent enzyme activities in the absence of, at unsaturating of and saturating conc. of lysine, respectively, we obtained a Hill coefficient $n_H=2.8$ and an inhibitor constant $I_{0.5}=0.044$ mM for L-lysine, where $I_{0.5}$ represents the lysine concentration giving half maximal inhibition (Fig. 8). In the later enzyme preparation including Sephacryl S-300 chromatography, the maximum inhibition of aspartokinase in the presence of saturating lysine concentration (10 mM) was increased to 83% and the $I_{0.5}$ was estimated to be 0.038 mM.

Protection of Aspartokinase by L-lysine against Thermal Denaturation

The thermal inactivation of aspartokinase followed first order reaction kinetics. As shown in Fig. 9, lysine stabilized the enzyme against inactivation. At 45°C and pH 7.5, the inactivation rate constant of aspartokinase in the absence of lysine was 0.0417 min^{-1}, however, in the presence of 1 mM L-lysine there were two inactivation rate constants, 0.0238 min^{-1} and 0.0054 min^{-1}. This suggests that possibly there are two forms of lysine sensitive aspartokinase with different thermal stabilities.

Discussion

The different end product inhibition patterns between rice seedling, milky-stage

leaf and leaf sheath aspartokinase were interesting (Table 1). In milky-stage rice, active protein biosynthesis (especially storage protein) clearly needs well balanced amino acid composition. Lysine-sensitive, threonine-sensitive and methionine-sensitive aspartokinase isozymes were probably all present at this stage since in leaf crude extract either one of these amino acids alone showed partial inhibition and all together produced nearly 90% inhibition of aspartokinase activity.

In the rice seedlings, only lysine sensitive aspartokinase was present and this aspartokinase was very sensitve to lysine, the concentration for half maximal inhibition was only 44 μM. (Fig. 7) This suggests that lysine pool size is particulary necessary under rigorous control at rice seedling development stage.

For comparison, the $I_{0.5}$ values of lysine for lysine sensitive aspartokinase from various sources were: *E. coli*, 0.2 mM to 0.48 mM (Shaw, 1977; Wampler and Westhead, 1968); maize, 13 μM (Cheshire and Miflin, 1975); carrot, 0.5 mM (Sakano and Komamine, 1978) *Lemna minor* L, 0.4 mM (Wong and Dennis, 1973); barley seedlings, 0.4–0.9 mM (Aarnes, 1977; Shewry and Miflin, 1977); wheat, 0.35 mM (Wong and Dennis, 1973).

It is well known that lysine content of corn is very low and maize aspartokinase happened to be the most sensitive enzyme to lysine inhibition ($I_{0.5}$=13 μM) (Cheshire and Miflin, 1975) so far reported in the literature. The rice lysine content is also relatively low compared with other crops except corn and its aspartokinase was also relatively sensitive to lysine inhibition ($I_{0.5}$=44 μM). The sensitivity of aspartokinase toward lysine inhibition seems to be a good indicator of lysine content. It has been shown recently that a variant cell line of maize resistant to lysine plus threonine possessed an aspartokinase activity that was less sensitive than the normal activity to inhibition by lysine and these cells contained increased amount of free lysine, methionine, threonine and isoleucine (Chaleff, 1981).

From the poor growth of rice callus tissue in methionine deficient media only when the media contained both threonine and lysine simultaneously, Furuhashi and Yatazawa (1970) proposed that lysine plus threonine inhibit methionine formation at aspartokinase possibly by either concerted feedback inhibition or simultaneous inhibition of two isozymes, each of which is sensitive to lysine and threonine, respectively. However, in our present studies only lysine sensitive aspartokinase was detected in rice seedlings and aspartokinase insensitive to end product inhibition might be present since 10 mM lysine inhibited only 47% of total aspartokinase activity (a in Table 1).

The crude extract of milky-stage rice leaf aspartokinase was sensitive to each of threonine, lysine and methionine, respectively. Threonine plus lysine inhibit less aspartokinase activity than either amino acid alone (c in Table 1). This suggests a antagonistic effect between amino acid bindings of aspartokinase. These studies do not support Furuhashi and Yatazawa hypothesis. Although it might be

possible that rice callus tissue aspartokinase has different control mechanism from rice-seedling and leaf enzyme, the interpretion of the inhibitory effects of amino acids on plant growth are complicated by the facts that they can cause inhibition of other processes. It has been shown that threonine is an effective inhibitor of nitrate uptake and methionine appears to be a general inhibitor of amino acid uptake (Miflin, 1977).

For rice-seedling aspartokinase, it is possible to put only lysine concentration under strict control by compartmentation of lysine sensitive aspartokinase. Thus the regulation of lysine biosynthesis could occur in a particular plastid without affecting the biosynthesis of threonine and/or methionine.

The basic properties of partially purified lysine sensitive aspartokinase from rice seedlings are similar to the enzymes of other sources. However, there are subtle differences, in addition to the sensitivity to lysine inhibition already mentioned, the rice lysine sensitive aspartokinase had a Hill coefficient of 2.8 (Fig. 8). This suggests that the lysine inhibition is cooperative and the enzyme should have at least three lysine binding sites. The Hill coefficients for wheat germ aspartokinase was 2.1 (Wong and Dennis, 1973).

The inhibition by lysine is competitive with respect to aspartate in maize aspartokinase. In contrast, it was noncompetitive with respect to either aspartate or MgATP in rice-seedling enzyme (Figs. 5 and 6). The Km values for rice aspartokinase were aspartate, 1.27 mM and MgATP, 0.55 mM, respectively (Figs. 5 and 6). These values were relatively low compared with aspartokinase isolated from other plants. For example, the Km values for aspartate were: barley seedling, 9 mM; wheat germ, 16.7 mM; maize, 9 mM (Cheshire and Miflin, 1975; Shewry and Miflin, 1977; Wong and Dennis, 1973).

That lysine can protect lysine-sensitive aspartokinase against thermal inactivation (Fig. 9) is a general phenomena of the lysine-sensitive aspartokinase reported so far (Cheshire and Miflin, 1975; Shaw, 1976). However, only the rice aspartokinase showed two thermal inactivation rate constants in the presence of saturating lysine concentration. Further molecular and kinetic studies on the completely purified enzyme would be needed to see if the rice lysine-sensitive aspartokinase exists in two conformations differentially stabilized by lysine or two isozymes with different lysine binding properties existed in the preparation.

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水稻天門冬胺酸激酶之性質及其調節機制

蕭 介 夫 顧 軍 瑛

中央研究院植物研究所

水稻幼葉之天門冬胺酸激酶僅能被 10 mM 離胺酸抑制47%，而其他測試胺基酸對此酵素無作用。乳熟期之水稻葉片天門冬胺酸激酶則可以被離胺酸，息寧胺酸，甲硫胺酸及異白胺酸以個別或組合方式抑制部份活性，但此時期水稻葉鞘部之天門冬胺酸激酶却不受任何測試胺酸之抑制。

我們將水稻幼葉之離胺酸敏感性天門冬胺酸激酶部份純化。以硫胺分劃，Sephacryl S-300 及 DEA-Sephadex A-50 管柱層析三步驟可得16倍純度之酵素。此酵素之最適反應 pH 值爲 7.5 而最適反應溫度爲 35°C，其對天門冬胺酸及 $MgATP^{2-}$ 之 Km 值各爲 1.37 mM 及 0.55 mM。其到達最大抑制一半所需之離胺酸濃度爲 44 μM 而其 Hill 係數爲2.8。此酵素之熱失活反應是一級反應。離胺酸具有保護酵素抗拒熱失活能力。在 pH 7.5 及 45°C 下，此酵素之熱失活反應常數爲 0.042 min^{-1}，而在 1 mM 離胺酸存在下，則有兩個反應常數，0.024 min^{-1} 及 0.005 min^{-1}。